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14. ABSTRACT

Objective: To increase our understanding of the molecular aberrations associated with endometrial carcinogenesis and the biologic mechanisms underlying the protective effect of oral contraceptive (OC) therapy. **Methods:** 1) Oligonucleotide microarray analysis was performed on a panel of endometrial cancers. 2) A subset of adenocarcinoma cases from the International DES Registry (IDESR) was analyzed for MSI 3) A case-control study of the CASH database was performed to evaluate the relationship between progestin potency and endometrial cancer risk. 4) An analysis of endometrium samples from cymologous macaques that were exposed to long term progestins was performed. 5) A clinical trial comparing progestin versus placebo is underway that will facilitate investigation of the effects of progestin exposure on the endometrial lining. **Results:** 1) Different histological types of endometrial cancer have unique genomic expression patterns. 2) The poor quality DNA from the majority of IDESR samples prohibited an adequate analysis of the case set. 3) A case-control study has suggested higher progestin- potency OCs may be more protective than lower progestin potency OCs among women with a larger body habitus. 4) Macaque studies have suggested that induction of apoptosis may be a mechanism underlying the chemoprotective effects of progestin on the endometrium. 5) Regulatory hurdles have resulted in delays in initiation of the clinical trial. Final FDA approval is expected within the next 3-4 months and the original objectives in the statement of work will be addressed.

15. SUBJECT TERMS
Endometrial cancer, hormone, prevention

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INTRODUCTION

Endometrial cancer is the most common type of gynecologic cancer in the United States and was estimated by the American Cancer Society to have been newly diagnosed among 40,000 American women in the year 2004 and lead to approximately 6500 cancer related deaths (1). Approximately 25% of all endometrial cancers occur in premenopausal women (2). Major advances in our understanding and treatment of endometrial cancer have occurred over the past decade, yet the frequency of this cancer in the general population has not been altered appreciably. Despite the known protective effect of oral contraceptives, little has been learned regarding the underlying mechanism. We believe that an understanding of the molecular profiles of endometrial cancers and the molecular events underlying the protective effect of oral contraceptives against endometrial cancer could facilitate the development of effective chemopreventives and significantly decrease the incidence of endometrial cancer in women.

BODY

Aim 1: To characterize and compare the molecular profiles of Type I endometrioid endometrial cancers, which often develop in an estrogen milieu, to that of Type II endometrial cancers. In addition, we will use microarray to examine the molecular changes in the endometrium associated with progestin exposure in order to gain insight into the biologic mechanism underlying the chemopreventive effect of the oral contraceptive pill (OCP).

Project 1: Objectives completed and data previously submitted with 2004 report. Data published this past year and listed in "Reportable Outcomes".

Project 2 (Pending): We recently requested a third no cost extension (NCE) for proposal No. 0155012, Award No. DAMD17-02-1-0183 that will involve Walter Reed Army Medical Center, Wake Forest University, and Evanston Northwestern. Our group has had significant delays in approval of the human trial outlined in Project 4, Study 1 ("The Chemoprotective Effects of Progestin on the Endometrial Lining"). We final received approval for HSRRB #A-11191.2b on 3/21/05. Unfortunately, during the interim of protocol review and approval locally and regionally (between 11/29/01 and 3/21/05), the manufacturer (Wyeth) of the study drug (Ovrette) had discontinued sales of the drug in the United States. After consideration of multiple options disclosed in last years NCE, we ultimately located a supplier of the drug and Analytical Research Laboratory (Oklahoma City, OK) and Northpointe Pharmacy (Oklahoma City, OK) were chosen as the testing laboratory and formulating pharmacy. Method development of the various analytical tests was commenced in January 2007 with formulation of active and placebo capsules beginning at the end of January. Release testing of the finished capsules will begin in May 2007 with stability testing beginning in June 2007. Results from the analytical tests and formulation process is scheduled to become available starting end of May 2007. At this point, we will have enough data to submit the IND for FDA approval. Once the IND is approved, the primary and secondary IRBs will need to acknowledge agreement with the application materials for final approval. We predict that the randomized placebo controlled trial will be underway by the end of 2007 at the latest.

Aim 2: To analyze vaginal and cervical adenocarcinomas, that have arisen in women exposed to DES in-utero, for methylation and mutation of PTEN and MLH1 in order to determine if estrogen induces genetic alterations in these tumors characteristic of Type I endometrioid carcinomas.
Although a pilot study aimed at an analysis of MSI in 7 cases from the International DES Registry was successful with repetitive attempts at DNA amplification, the analysis of the entire set was not successful presumably secondary to the quality of the DNA which reflects the old age of the specimens and the various methods that were used in their preservation. Less than 50% of the samples amplified at any one of the markers making the data inadequate for designation of MSI status. Significant amounts of material from the Transplacental Registry were used unsuccessfully to complete the work on the microsatellite instability. Acquisition of additional material to further evaluate alterations in either mismatch repair genes (causative of MSI) or PTEN was not an option. The inability to pursue this aim further was previously described in the 2006 annual report.

Aim 3: Using data from the Centers for Disease Control Cancer and Steroid Hormone Study, we will determine if the protective effect of OCP's against endometrial cancer are impacted by the progestin or estrogen potency of OCP formulations.
Objectives completed and data previously submitted with 2004 report. Published manuscript listed in "Reportable Outcomes"

Aim 4: To test the hypothesis that the oral contraceptives and hormone replacement therapy progestins provide a chemoprotective effect against endometrial cancer through induction of apoptosis, PTEN, and TGF-beta in the endometrium.
Epidemiological studies have demonstrated that OCP use lowers the risk of subsequent endometrial and ovarian cancer. Although the biologic mechanism(s) underlying the protective effect of OCP's on the risk of both of these cancers have not been well defined, there is evidence to suggest that biologic effects related to the progestin component may underlie the cancer preventive effects of the OCP. Recent studies have reported the progestin-mediated activation of apoptosis in endometrial cancer cell lines and endometrial hyperplasias. The finding that progestin activates the apoptosis pathway in endometrial cells raises the possibility that this may be a major mechanism underlying the therapeutic effect of progestins against endometrial hyperplasia. Similarly, our group has found that progestins markedly activate both apoptosis and TGF-beta expression in the ovarian epithelium leading to the hypothesis that progestins may act as chemopreventives for ovarian cancer. It is interesting that tumors arising from the ovary and endometrium share common epidemiological risk factors, and that both the endometrium and ovarian surface epithelium share a common embryological precursor. It is thus plausible that progestins activate similar molecular pathways relevant to cancer prevention in both of these organ sites. Recent evidence suggests that expression *PTEN* appears to be upregulated in the secretory phase of the menstrual cycle. It is plausible that the chemopreventive effects of OCP's are mediated through overexpressed *PTEN* with resultant suppression of cell cycle progression and activation of apoptosis in endometrial cells.

- The short-term effects of progestins on apoptosis as well as the expression of *PTEN* and TGF-β in the endometrium will be evaluated using uterine specimens collected from patients enrolled in a double-blinded prospective randomized trial. See Aim1, project 2 for explanation of delays in deliverables and current "no cost extension status".

- The long term effects of progestins on apoptosis as well as the expression of PTEN and TGF-b in the endometrium were evaluated using uterine specimens from cynomolgus macaques (80 premenopausal and 130 postmenopausal) previously part of a three-year randomized trial designed to evaluate the effects of the combination oral contraceptive pill and hormone replacement therapy on reproductive organs.

PREMENOPAUSAL

Statistical analysis of the data involving the premenopausal colony presented with the last report did not reveal any statistical relationships. We subsequently elected to discontinue further analysis of these tissue specimens and did not elect to prepare these findings for publication.

POSTMENOPAUSAL COLONY

Epidemiological, animal, and human data suggest that progestins are potent endometrial cancer preventive agents. In the ovary, progestins have been hypothesized to confer a cancer preventive effect via activation of apoptosis and modulation of Transforming Growth Factor-Beta (TGF-β) in the ovarian surface epithelium. Given that the ovarian epithelium and endometrium share a common embryologic origin and similar reproductive and hormonal risk factors for malignancy, we sought to test the hypothesis that progestins confer biologic effects in the endometrium similar to that shown in the ovary.

Methods:

Postmenopausal female macaques (N=78) were randomized into four groups to receive a diet for 35 months containing no hormone, versus Premarin (CEE), Provera (MPA), or CEE plus MPA given in a continuous fashion. At study termination, the endometrium was examined immunohistochemically for evidence of apoptosis using antibody to activated caspase-3, and for expression of Ki67 and the TGF-β1, 2, and 3 isoforms.

Results:

Effect of Hormone Treatment on Apoptosis in Endometrium

In general, few apoptotic cells were noted in the endometrium from either the control or CEE-only-treated monkeys. In contrast, progestin treatment with MPA, either in combination with CEE or alone was associated with significant increases in apoptosis in both glandular and stromal cells (Figure1).

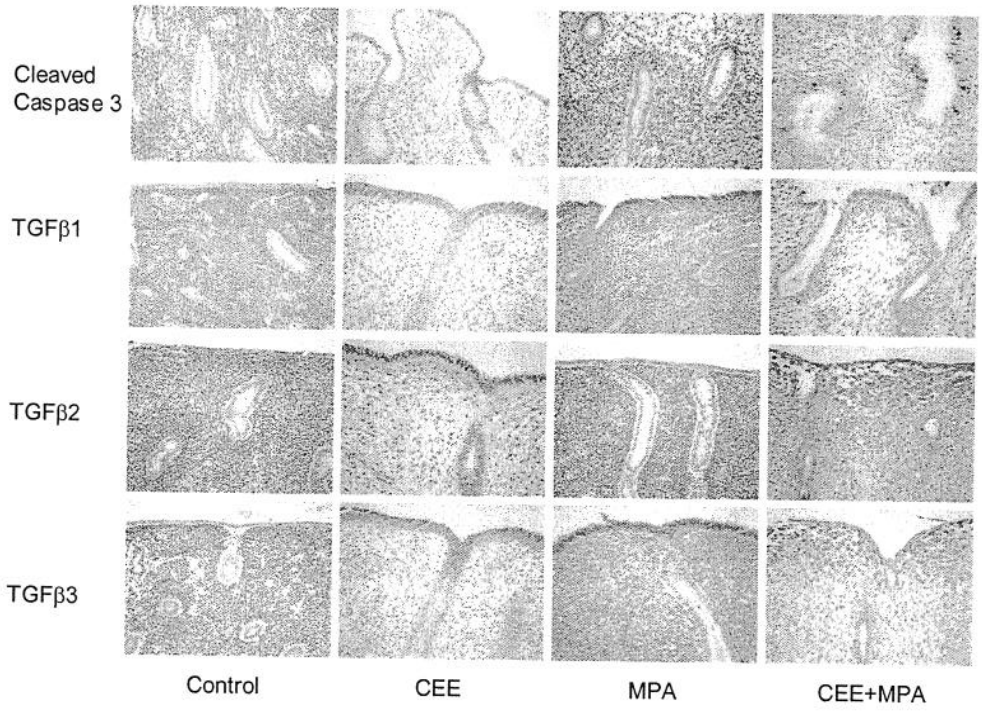

Figure 1. Representative sections from each of the treatment groups, immunostained for activated caspase-3 and the TGF-β isoforms.

Percentages of caspase-positive superficial glandular and basal glandular epithelial cells were 3-5 fold higher in CEE+MPA-treated animals compared to all others ($p<0.05$). In addition, caspase-3-expressing cells were 6 times more numerous in the superficial stroma of animals treated with MPA alone, relative to other groups ($p<0.0001$; Figure 2).

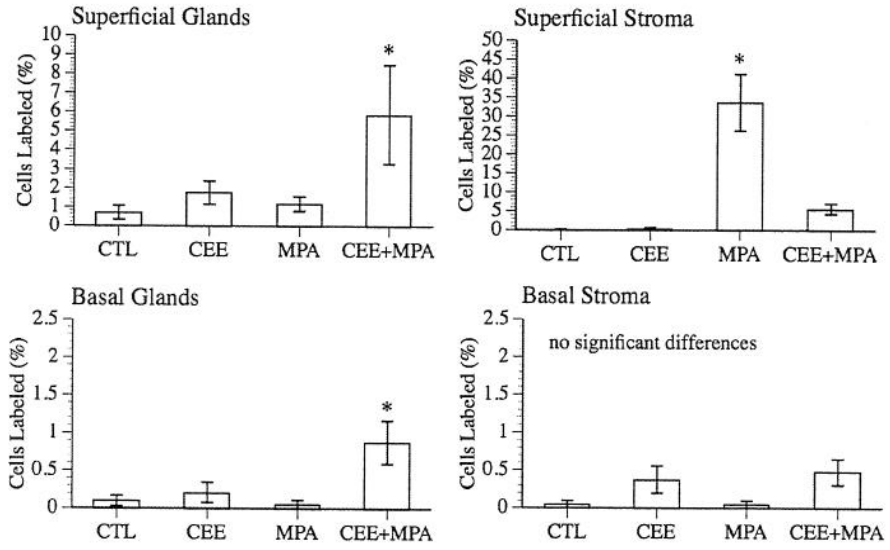

Figure 2: Cleaved caspase-3 immunostaining expressed as a percentage of cells counted in glandular and stromal compartments of the superficial and basal endometrium. Asterisks indicate groups whose means differ from controls at $p<0.05$

Effect of Hormone Treatment on Proliferation in Endometrium

Expression of the proliferation marker Ki67 was strongly induced in the glandular epithelium of the functionalis by treatment with CEE. This effect was antagonized by the addition of MPA to the treatment regimen. For both CEE and CEE+MPA, proliferation was increased in the stromal compartment of the functionalis. The basalis was relatively unresponsive, with proliferation not being significantly increased in either the epithelium or the stroma by any treatment (Figure 3).

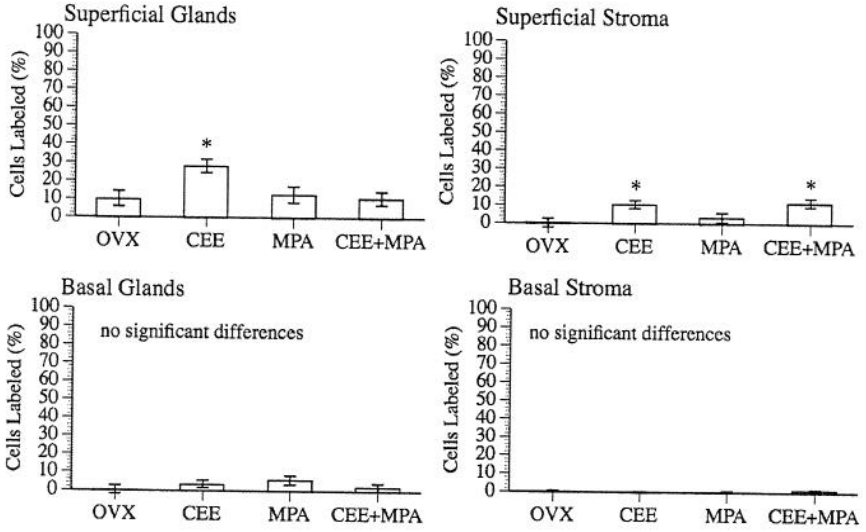

Figure 3. Ki67 immunostaining expressed as percent cells labeled. Asterisks indicate groups whose means differ from controls at $p<0.05$

Effect of Hormone Treatment on Expression of TGF-β

Expression of the TGF-β isoforms varied throughout the endometrium, relative to both treatment group and location within the endometrium (basalis versus functionalis). In the endometrial glandular compartment, expression of TGF-β1 was scant in both the untreated monkeys as well as those receiving MPA alone. In contrast, treatment with CEE was associated with a marked increase in the expression of TGF-β1 in basal glands as compared to controls (p=0.009). The addition of MPA to CEE abrogated the CEE-related increase in TGF-β1 expression by 50% (p= 0.039). Stromal expression of TGF-β1 was absent across all treatments (Figures 1 and 4).

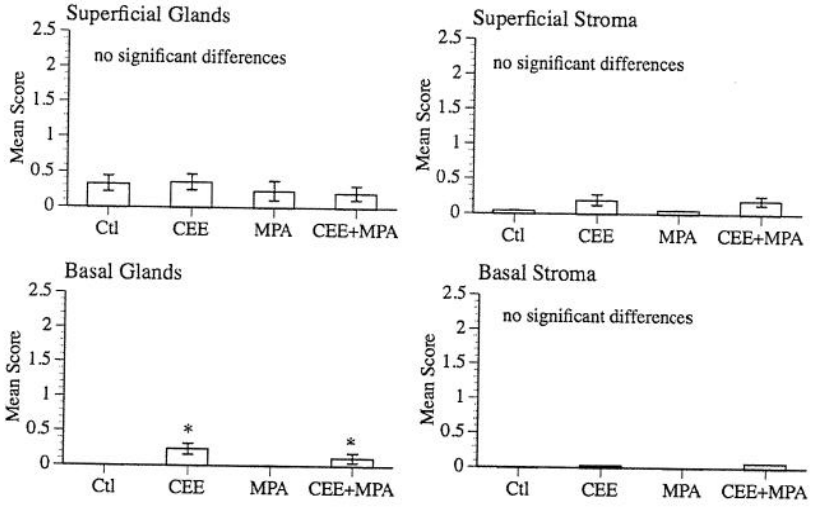

Figure 4: TGF-β1 immunostaining expressed as mean staining score in surface epithelium and glandular and stromal compartments of the superficial and basal endometrium. Asterisks indicate groups whose means differ from controls at $p<0.05$. In Basal Glands, immunostaining in the CEE and CEE+MPA groups was greater than controls (p= 0.009 and 0.039, respectively).

Expression of TGF-β2 was scant in the epithelial compartment, and not affected by treatment. However, expression of TGF-β2 was markedly increased in the superficial endometrial stroma of animals treated with CEE+MPA relative to all other groups ($p < 0.01$; Figures 1 and 5).

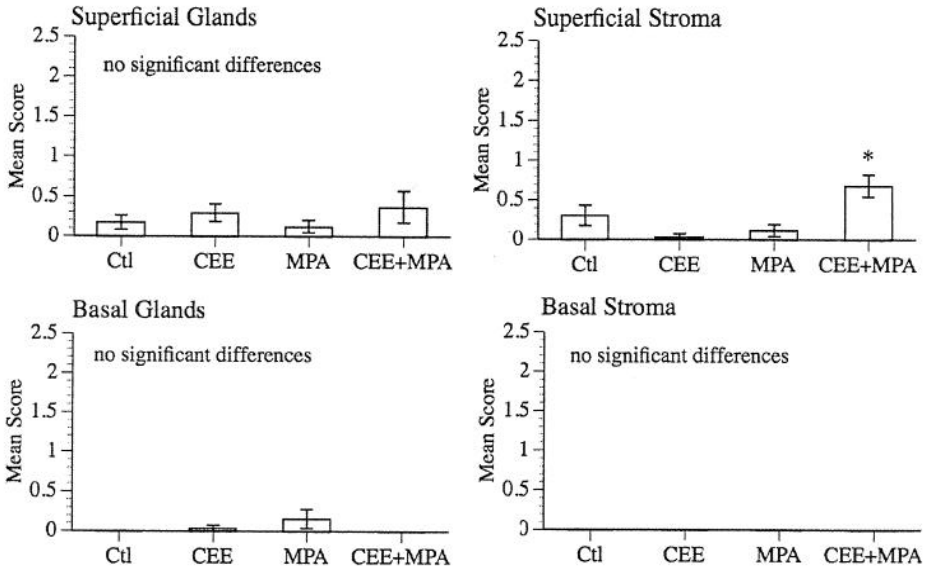

Figure 5: TGF-β2 immunostaining expressed as mean staining score in surface epithelium and glandular and stromal compartments of the superficial and basal endometrium. Asterisks indicate groups whose means differ from controls at $p<0.05$

For the TGF-β3 isoform, expression in the glandular compartment was variable. MPA-treated animals had lower superficial glandular TGF-β3 than either controls ($p<0.01$) or CEE-treated animals ($p=0.004$). TGF-β3 was not seen in basal glands of control and CEE+MPA-treated animals, but was detectable in CEE-treated animals ($p<0.005$). In contrast, TGF-β3 expression in superficial stroma was markedly elevated in CEE+MPA treated animals, differing from all other groups ($p<0.0001$; Figures 1 and 6). Among CEE+MPA-treated animals, those lacking TGF-β3 in the superficial stroma had the greatest amount of superficial glandular Ki67 expression ($p=0.032$). Among controls, those animals expressing the highest score for TGF-β2 in the superficial stroma had higher levels of superficial glandular Ki67 expression ($p = 0.02$).

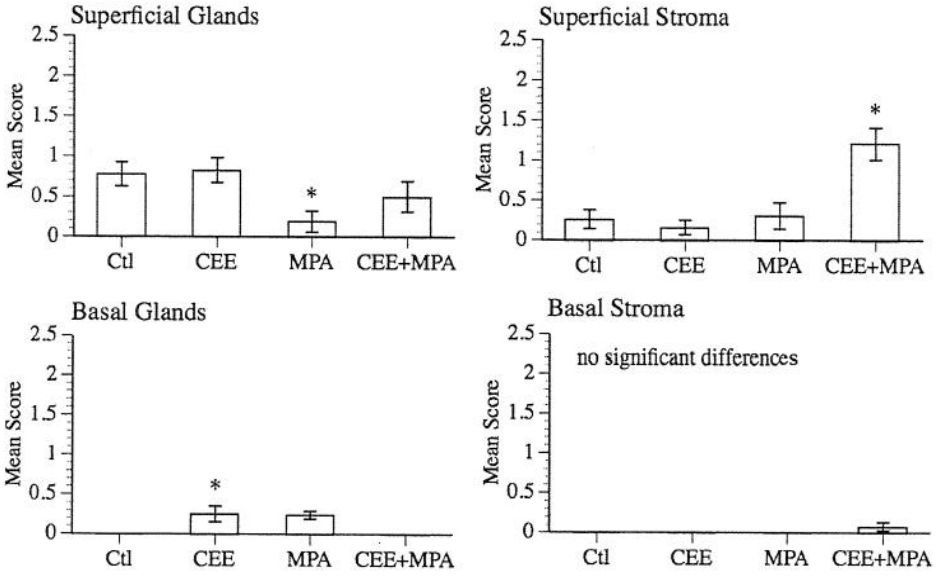

Figure 6: TGF-β3 immunostaining expressed as mean staining score in surface epithelium and glandular and stromal compartments of the superficial and basal endometrium. Asterisks indicate groups whose means differ from controls at $p<0.05$. In Superficial Glands, immunostaining in the MPA group was significantly lower than both control and CEE.

CONCLUSION: Progestin treatment activates chemopreventive biologic effects in the endometrium that are similar to those in the ovarian surface epithelium. These data may facilitate identification of a chemopreventive approach that dramatically lessens the risk of both uterine and ovarian cancer.

KEY RESEARCH ACCOMPLISHMENTS

- Aim 1: Identified genes that are differentially expressed between endometrioid and papillary serous endometrial carcinoma and determined that histology can be predicted on the basis of gene expression in approximately 90% of cases. Identified additional genes that are differentially expressed in endometrial cancer vs. normal endometria. Confirmed that microsatellite stable endometrial cancers have unique gene expression profiles compared to those with microsatellite instability
- Aim 3: Established that progestin containing oral contraceptives (OCs) are associated with a decreased endometrial cancer risk and that higher progestin- potency OCs may be more protective than lower progestin potency OCs among women with a larger body habitus.
- Aim 4: Completed analysis of apoptosis and TGF using endometrium specimens from macaques exposed to various hormonal regimens and found evidence to explain the chemoprotective effects of progestin on the post-menopausal endometrial lining

REPORTABLE OUTCOMES (since last report)

- Rodriguez GC, Rimel BJ, Watkin W, Turbov J, Barry C, Maxwell GL, Cline JM: Progestin Treatment Induces Apoptosis and Modulates TGF-β in the Uterine Endometrium. Plenary presentation at the annual Society of Gynecologic Oncology, San Diego 2007. Manuscript attached and being submitted for publication

CONCLUSIONS

Different histological types of cancer have genomic expression patterns that reflect unique pathways of carcinogenesis. Likewise, cancers characterized by microsatellite instability result in the expression of genes most likely to be affected by alterations in mismatch repair. As we improve our understanding of the alterations that accompany endometrial carcinogenesis, it is likely that future chemopreventives may be developed for several types of endometrial cancer, each of which develops by specific pathways. In regards to contemporary chemopreventive options, an analysis of data from the CDC CASH database has suggested that a greater protective effect against endometrial cancer may be associated with high progestin potency OCs, particularly in patients with a larger body habitus. Higher potency progestin containing OCs should be considered in forthcoming endometrial cancer prevention trials particularly if other studies suggest a greater risk reduction associated with heavier women that are at highest risk for endometrial cancer. Using a macaque model, we have determined that the mechanisms behind the chemoprotective effects of progestin containing hormonal regimens in post-menopausal patients appear to be in part related to induction of apoptosis. We look forward to evaluation of the short term effects of

progestin containing hormonal formulations in our clinical trial evaluating the short term effects of progestin on the endometrium lining using both a targeted analysis of apoptosis as well as an assessment of global gene expression using oligonucleotide microarray.

REFERENCES

1. Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ, Thun MJ: Cancer statistics, 2004. CA Cancer J Clin 2004;54:8-29.
2. Gallup DG, Stock RJ: Adenocarcinoma of the endometrium in women 40 years of age or younger. Obstet Gynecol 1984;64:417

Progestin Treatment Induces Apoptosis and Modulates TGF-β in the Uterine Endometrium

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Abstract

OBJECTIVE: Epidemiological, animal, and human data suggest that progestins are potent endometrial cancer preventive agents. In the ovary, progestins have been hypothesized to confer a cancer preventive effect via activation of apoptosis and modulation of Transforming Growth Factor-Beta (TGF-β) in the ovarian surface epithelium. Given that the ovarian epithelium and endometrium share a common embryologic origin and similar reproductive and hormonal risk factors for malignancy, we sought to test the hypothesis that progestins confer biologic effects in the endometrium similar to that shown in the ovary.

METHODS: Postmenopausal female macaques (N=78) were randomized into four groups to receive a diet for 35 months containing no hormone, versus Premarin (CEE), Provera (MPA), or CEE plus MPA given in a continuous fashion. At study termination, the endometrium was examined immunohistochemically for evidence of apoptosis using antibody to activated caspase-3, and for expression of Ki67 and the TGF-β1, 2, and 3 isoforms.

RESULTS: Percentages of caspase-positive superficial glandular and basal glandular epithelial cells were 3-5 fold higher in CEE+MPA-treated animals compared to all others ($p<0.05$). Caspase expressing cells were 6 times more numerous in the superficial stroma of animals treated with MPA alone, relative to other groups ($p<0.0001$). Induction of superficial glandular cell apoptosis in the CEE+MPA-treated group was associated with a

dramatic increase in expression/production of TGF-β2 and TGF-β3 in the stromal compartment of the endometrium functionalis ($P < 0.001$).

CONCLUSION: Progestin treatment activates chemopreventive biologic effects in the endometrium that are similar to those in the ovarian surface epithelium. These data may facilitate identification of a chemopreventive approach that dramatically lessens the risk of both uterine and ovarian cancer.

Introduction

Approximately 150,000 new cases of endometrial cancer are diagnosed every year worldwide, making endometrial cancer the fifth leading cancer in women globally. In the United States it is estimated that 41,200 new cases of endometrial cancer will be diagnosed in 2006. (1,2) A number of risk factors for endometrial cancer have been identified including nulliparity, early menarche, late menopause, high body mass index, and the administration of unopposed estrogen for the treatment of climacteric symptoms. (3-9)

In the past, endometrial cancer has not garnered as much attention as ovarian cancer. This may be secondary to the better overall prognosis of endometrial cancer, as the majority of women diagnosed with endometrial cancer are cured. Yet, stage for stage, survival rates for women with endometrial cancer are similar to those for ovarian cancer. (1) In addition, highly effective treatments for advanced stage endometrial cancer are lacking. With an aging and increasingly obese population in the U.S., the incidence and mortality from endometrial cancer is likely to become a worsening public health problem thereby providing a strong rationale for the development of effective methods to prevent the disease. (10)

A strong body of evidence suggests that progestins are highly effective endometrial cancer preventive agents. In premenopausal women, use of oral contraceptives (OCs) that contain both an estrogen and a progestin significantly lowers

subsequent endometrial cancer risk. Use of OCs for a period of at least 12 months confers as much as a 30-50% reduced risk of endometrial cancer, a protective effect which lasts for 10-20 years. (11-15) In addition, progestin-potent OCs appear to have enhanced endometrial cancer protective effects compared to OCs containing weak progestins (16-17), and use of progestin-releasing intrauterine devices which release potent dosages of progestins locally in the endometrial cavity leads to marked reduction in endometrial cancer risk. (18, 19) In menopausal women, the addition of a progestin to estrogen replacement therapy decreases the risk of precancerous endometrial hyperplasias and abrogates the cancer-causing effect of exogenous estrogen (4, 9), suggesting that progestins have a chemopreventive biologic effect on the endometrium. Finally, high-dose progestin therapy has been shown to reverse preexisting PTEN-inactivated endometrial latent precursors, as well as endometrial hyperplasias and even low grade endometrial cancers in some women. (20-22)

The biologic mechanism(s) that underlie the chemopreventive effect of progestins on the endometrium have not been well characterized. In the ovary, progestins have been shown to induce programmed cell death and differentially regulate Transforming Growth Factor-Beta (TGF-β) in the ovarian surface epithelium, leading to the hypothesis that progestin-mediated biologic effects lead to the arrest or reversal of carcinogenesis via clearance of genetically damaged ovarian epithelial cells. This biologic mechanism may explain the marked reduction in ovarian cancer risk associated with OC use. (23) It is interesting that cancers arising from the ovary and endometrium share common epidemiological risk factors, including marked risk reduction associated with use of OCs (24-35), and that both the endometrium and ovarian surface epithelium share a common

embryological precursor. (36) It is thus plausible that progestins activate similar molecular pathways relevant to cancer prevention in both of these organ sites. To test this hypothesis, we examined the endometria of primates treated with hormonal interventions including progestin for evidence of induction of apoptosis and for regulation of expression of the TGF-β isoforms.

Materials and Methods

Animals/Randomization

For this study, we used 78 female adult cynomolgus macaques (*Macaca fascicularis*), with an average age of 7.5 years at the study's end. The cynomolgus macaque is an excellent animal model for yielding experimental results that are pertinent to human reproductive biology. This nonhuman primate has a 28-day menstrual cycle that is similar to that of humans. (38-40) The study was a prospective, randomized, controlled trial designed for the primary endpoint of evaluating the effects of post menopausal estrogens and progestins on the on the cardiovascular system, breast, and reproductive tracts. The macaques previously had undergone bilateral oophorectomy three months prior to commencement of the study. The study was approved by the Animal Care and Use Committee at the Wake Forest University School of Medicine, Winston-Salem, NC.

The macaques were randomly assigned to receive one of four hormonal interventions for three years; control (n= 19), Premarin (CEE) (n=24) [Wyeth Ayerst, St. Davids, PA], Provera (MPA) (n=18), [Wyeth Ayerst, St. Davids, PA], and CEE and

MPA combined (n= 17). Test compounds were administered in the diet, at human equivalent doses on a caloric basis to 0.625 mg/woman/day for CEE, and 2.5 mg/woman/day for MPA. The duration of treatment was 36 months. The base diet was modeled on a typical moderately atherogenic North American diet (40% of calories from fat, and 0.2 mg/kcal cholesterol). Doses were scaled on the basis of caloric intake, which takes into account species differences in metabolic rate; this is the generally accepted way to achieve dosages comparable to those in women. This regimen was given on a continuous basis until the end of the study. At the conclusion of the trial, animals were humanely euthanized by sedation with ketamine (10 mg/kg) and administration of 100 mg/kg pentobarbital intravenously. Complete necropsy examinations were performed including examination of multiple organ systems, as reported previously. (41)

Tissue Preparation and Immunohistochemistry

From each animal in the study, the uterus was fixed in 4% formaldehyde for 24 hours and then stored at 4 degrees Celsius in 70% ethanol. Thereafter, uterine sections were trimmed to 3 mm thickness, and embedded in paraffin.

Immunostaining: Briefly, 5um full sections through the uterine wall (endometrium to the serosa) were mounted on charged slides, and tissues were immunostained using our previously published methods. (23, 42) Antigen retrieval with heat and citrate buffer (pH 6.0) was used for all antibodies. Appropriate quenching procedures (heat treatment and peroxidase treatment) were used to remove endogenous enzymatic activity. Staining for apoptosis was done using a monoclonal antibody to cleaved caspase-3 (2 μg/mL, Cell-

Signaling Technology, Beverly, Mass); sections of benign macaque early menstrual phase endometrium were used as a positive control. Staining for TGF-β isoforms was done using antibodies specific for TGF-β1 (0.67 μg/mL anti- TGF-β1 antibody catalog #SC-146, Santa Cruz Biotechnology, Santa Cruz, CA.), TGF-β2 (4μg/ml anti- TGF-β2 antibody, catalog #SC-90 Santa Cruz Biotechnology, Santa Cruz, CA), and TGF-β3 (0.25 μg/mL anti TGF-β3 antibody, catalog # SC-83, Santa Cruz Biotechnology, Santa Cruz, CA). For TGF-β staining, normal human umbilical cord was used as a positive control. Staining for proliferating cells was done using a monoclonal antibody to Ki67 (1.25 μg/mL clone MM1, Novocastra Laboratories, Newcastle-Upon-Tyne, UK). For caspase and Ki67 staining, the enzyme detection system used alkaline phosphatase and the chromogen used was Vector Red (Vector Laboratories, Burlingame, CA); for TGF-β staining, the enzyme system used was peroxidase and the chromogen was diaminobenzidine (Dako Carpinteria, CA). Sections were counterstained with Mayer's hematoxylin.

Caspase and Ki67-stained cells were quantified by counting the percentages of stained and unstained cells in sections by an observer blinded to treatments. The percentages of immunopositive uterine glandular cells as well as stromal cells in both the basalis and functionalis were quantitated. TGF-β staining was also assessed in glands and stroma of the functionalis and basalis. Staining was graded for each cell type and endometrial location as 0 (unstained) to 3+ (heavily stained) by three reviewers, including a veterinary pathologist (JMC). All observers were again blinded to treatment.

Statistical Analysis

Both the percentage of caspase-3-expressing cells and the semiquantitative assessments of immunostaining for TGF-β isoforms were analyzed for treatment group as continuous variables using analysis of variance and a 2-tailed significance level of 0.05. Semiquantitative measures were also analyzed as ordinal data (analysis not shown). Pairwise t-tests were used to identify specific group differences if the overall analysis of variance showed significance. Within-group correlation analysis was done using Pearson correlation coefficients to identify associations between dependent variables.

Results

Effect of Hormone Treatment on Apoptosis in Endometrium

In general, few apoptotic cells were noted in the endometrium from either the control or CEE-only-treated monkeys. In contrast, progestin treatment with MPA, either in combination with CEE or alone was associated with significant increases in apoptosis in both glandular and stromal cells (Figure1). Percentages of caspase-positive superficial glandular and basal glandular epithelial cells were 3-5 fold higher in CEE+MPA-treated animals compared to all others ($p<0.05$). In addition, caspase-3-expressing cells were 6 times more numerous in the superficial stroma of animals treated with MPA alone, relative to other groups ($p<0.0001$; Figure 2).

Effect of Hormone Treatment on Proliferation in Endometrium

Expression of the proliferation marker Ki67 was strongly induced in the glandular epithelium of the functionalis by treatment with CEE. This effect was antagonized by the addition of MPA to the treatment regimen. For both CEE and CEE+MPA, proliferation was increased in the stromal compartment of the functionalis. The basalis was relatively

unresponsive, with proliferation not being significantly increased in either the epithelium or the stroma by any treatment (Figure 3).

Effect of Hormone Treatment on Expression of TGF-β

Expression of the TGF-β isoforms varied throughout the endometrium, relative to both treatment group and location within the endometrium (basalis versus functionalis). In the endometrial glandular compartment, expression of TGF-β1 was scant in both the untreated monkeys as well as those receiving MPA alone. In contrast, treatment with CEE was associated with a marked increase in the expression of TGF-β1 in basal glands as compared to controls ($p=0.009$). The addition of MPA to CEE abrogated the CEE-related increase in TGF-β1 expression by 50% ($p= 0.039$). Stromal expression of TGF-β1 was absent across all treatments (Figures 1 and 4).

Expression of TGF-β2 was scant in the epithelial compartment, and not affected by treatment. However, expression of TGF-β2 was markedly increased in the superficial endometrial stroma of animals treated with CEE+MPA relative to all other groups ($p <0.01$; Figures 1 and 5). For the TGF-β3 isoform, expression in the glandular compartment was variable. MPA-treated animals had lower superficial glandular TGF-β3 than either controls ($p<0.01$) or CEE-treated animals ($p=0.004$). TGF-β3 was not seen in basal glands of control and CEE+MPA-treated animals, but was detectable in CEE-treated animals ($p<0.005$). In contrast, TGF-β3 expression in superficial stroma was markedly elevated in CEE+MPA treated animals, differing from all other groups ($p <0.0001$; Figures 1 and 6). Among CEE+MPA-treated animals, those lacking TGF-β3 in the superficial stroma had the greatest amount of superficial glandular Ki67 expression ($p=0.032$). Among controls, those animals expressing the highest score for TGF-β2 in

the superficial stroma had higher levels of superficial glandular Ki67 expression ($p = 0.02$).

Discussion

In this study, we have characterized the complex opposing effects of estrogen and progestin on proliferation, apoptosis and TGF-β signaling in the uterine endometrium. In an ovariectomized menopausal primate model, we demonstrate that estrogen induces proliferation in superficial glands and increases expression of the TGF-β1 and TGF-β3 isoforms in basal glands, while having no impact on apoptosis. In contrast, progestin inhibits proliferation and differentially regulates expression of TGF-β, causing a decrease in expression of TGF-β3 in the superficial endometrial glands, while dramatically increasing expression of the TGF-β2 and TGF-β3 isoforms in the superficial stroma. In addition, progestin abrogated the estrogen-related increase in glandular expression of TGF-β3, and markedly induced apoptosis in both endometrial glands and superficial stroma. Areas of marked apoptosis in both endometrial glands and superficial stroma associated with progestin treatment occurred concurrently with marked increased expression of TGF-β2 and TGF-β3 in the superficial stroma, suggesting that these two biologic events may be related. Finally, progestin-mediated apoptotic effects in endometrial glands occurred only in the presence of estrogen, suggesting the requirement for estrogen priming, whereas estrogen was not required for progestin to induce marked apoptotic effects in the superficial endometrial stroma.

Overall, our findings are consistent with published data regarding the effect of progestins on apoptosis and TGF-β signaling in the endometrium. Progestins have been

shown to induce apoptosis in endometrial glands and stroma, an effect that is modulated by a number of well known apoptosis-related proteins. (22, 43-48) In the mouse, an increase in the Bax/BCL-2 ratio and associated increase in apoptosis has been shown in endometrial stromal cells in response to progestins, suggesting that progestin-mediated apoptotic effects contribute to the remodeling changes in the uterus associated with decidualization. (44) In women, upregulation of Fas/FasL expression has been shown in endometrial cells in response to progestins, and conversely dysregulation of Fas/FasL expression in hyperplastic endometrium may underlie failure to respond to progestin therapy. (46) In addition, progestins have been shown to increase the Bax/Bcl-2 ratio and induce apoptosis *in vivo* in women undergoing treatment for endometrial hyperplasia. Moreover, changes in BAX, BCL-2 and apoptosis have been shown to be correlated with the potency of progestin therapy, and in turn with the likelihood of response to therapy. (22). Thus, activation of apoptosis may be an important mechanism underlying the chemopreventive effects of progestins in the endometrium. With regard to TGF-β, both TGF-β2 and TGF-β3 have been shown to be increased in the endometrium during the secretory phase or in response to progestins *in vivo*, (49-52) whereas expression of TGF-β1 was unchanged. (49) Similarly, in the primate endometrium in response to MPA, we found overall levels of TGF-β2 and TGF-β3 to be dramatically increased, without a significant effect on TGF-β1.

Our findings in the endometrium bear remarkable similarities to what we described previously in the ovary. Previously, we have shown that progestin induces apoptosis and differentially regulates expression of TGF-β in the ovarian surface epithelium. (23) We observed a four-fold increase in apoptosis in the ovarian epithelium

in primates treated with the progestin levonorgestrel. This was associated with a significant increase in expression in the ovarian epithelium (OSE) of the TGF-β2 and 3 isoforms and a decrease in expression of TGF-β1. In the endometrium, our findings were similar except for the absence of an impact of progestin on TGF-β1 expression, although the addition of MPA to CEE appeared to lessen the degree of CEE-related induction of TGF-β1, a trend similar to our prior findings in the ovarian surface epithelium. In addition, in the OSE, changes in expression of TGF-β were localized specifically in the OSE, whereas the predominant change in expression of TGF-β in the endometrium is primarily in the superficial stroma. Thus, in the ovary, local changes in expression of the TGF-β isoforms may have an autocrine and paracrine effect on the OSE, whereas in the endometrium, stromal changes in expression of TGF-β may have an autocrine effect in stromal cells, and a paracrine effect in adjacent glandular cells. In addition, in the OSE, increases in expression of TGF-β2 and 3 were noted with progestin administered alone, whereas in the endometrium induction of expression of these two isoforms in the superficial stroma by progestin required the presence of estrogen. Thus, it is possible that optimal response to progestin in the endometrium requires estrogen priming, whereas this is not a necessary prerequisite in the OSE. Conversely, it is possible that estrogen priming is necessary for optimization of progestin effects on TGF-β in both sites. Our earlier study evaluating progestin in the OSE used pre-menopausal monkeys who had not undergone ovariectomy and thus received progestin in the setting of an adequate endogenous estrogenic milieu. Alternatively, the differences noted between the two studies may have been related to differences in the progestins used, in that the prior study used the gonane progestin levonorgestrel, in contrast to the pregnane progestin MPA used

in the current study. Nonetheless, the finding that progestins have similar biologic effects in the OSE and endometrium suggests a common biologic mechanism that may underlie the cancer preventive effect of progestins at both sites.

There is mounting evidence that differential regulation of peptide growth factors by steroid hormones contributes to the diverse end effects of these hormones in target tissues. Among the growth factors, TGF-β has been shown to be differentially regulated by estrogens, retinoids, androgens, and vitamin D compounds. In bone, raloxifene increases the expression of

TGF-β3 while having no effect on the expression of TGF-β 1 and TGF-β2. (53) In cells derived from the breast, estradiol decreases the expression of TGF-β2 and TGF-β3 while having

no effect on the expression of TGF-β1 (54), whereas tamoxifen has been shown to increase the expression of TGF-β1. (55) In chondrocytes, vitamin D increases the expression of TGF-β2

and decreases the expression of TGF-β1 and TGF-β3. (56) Glucocorticoids differentially regulate TGF-β in healing wounds, leading to the suppression of TGF-β1 and TGF-β2 and the increased expression of TGF-β3. (57) In the palates of mice, retinoids have been shown to decrease the expression of TGF-β1 while having no effect on the expression of other TGF-β isoforms (58), whereas in keratinocytes, induction of TGF-β2 is a major mechanism underlying the biologic effects of retinoids. (59,60) Finally, in the male accessory organs, androgen withdrawal is associated with both apoptosis and differential regulation of TGF-β. (61) Thus, the TGF-β isotypes appear to be differentially regulated in a tissue-specific manner. Although the

mechanism underlying the complex regulation of TGF-β by hormones is not completely understood, differences in the promoter region among the TGF-β isoforms or in post-transcriptional events may be means by which TGF-β is differentially regulated. (62-65)

The design of our study does not allow us to prove a causal relationship between changes in TGF-β expression and apoptosis in endometrial glands and stroma. However, it is possible that the two are related. Among the growth factors, TGF-β has been implicated as an important regulator of apoptosis. (66-68) In addition, in cells derived from the ovarian and uterine epithelium, TGF-β has been shown to induce apoptosis. (69-72) Furthermore, in hormone sensitive tissues such as the breast and prostate, TGF-β has been shown to mediate the apoptotic effects of steroid hormones, including the antiestrogens, retinoids, and vitamin D. (73-84) Finally, TGF-β is related to müllerian inhibitory factor (MIF), a peptide that causes complete apoptotic regression of the müllerian system (the precursor to the uterus, fallopian tubes, and upper vagina) in the developing male embryo *in vivo*, (85-88) as which has been shown to inhibit growth and induce apoptosis *in vitro* in cells derived from the ovarian and uterine epithelium. (89-93) Given the marked inhibitory effect that the members of the TGF-β family have on the müllerian system, it is interesting to speculate that the uterine endometrium may be uniquely susceptible *in vivo* to undergoing apoptosis in response to TGF-β and that agents that selectively regulate TGF-β in the endometrium may be potent apoptosis-inducing agents and cancer preventive agents.

A growing body of laboratory and *in vivo* evidence has implicated TGF-β as a potent tumor suppressor and cancer preventive agent. (94-99) Transgenic mice that have a constitutively active form of TGF-β1 are resistant to 7,12-dimethylbenz[a]anthracene-

induced mammary tumors (100). Conversely, mice with heterozygous deletions of one copy of the

TGF-β gene have an increased susceptibility to chemical carcinogenesis (101). In humans, mutations have been described in the TGF-β signaling pathway in a variety of tumors, including

cancers of the colon, gastric, pancreatic, and uterine tissues, and cancers of lymphoid organs. (97, 102-106) Furthermore, a number of cellular oncogenes are known to inhibit TGF-β activity. Finally, TGF-β has been implicated as a mediator of the biologic effects of a number of chemopreventive agents, including tamoxifen, which induces expression of TGF-β1 in stromal

cells in the breast (54), as well as retinoids, which induce TGF-β in the prostate and respiratory tract. (96) Taken together, these data provide compelling evidence that TGF-β plays an important role as an inhibitor of carcinogenesis and that agents which exploit this pathway may have utility for the chemoprevention of cancer.

Similarly, the apoptosis pathway also holds great promise as a target for chemoprevention. Activation of apoptosis leads to the efficient disposal of cells that have undergone irreparable genetic damage and which are prone to neoplastic transformation. (107) It is thus a key molecular pathway for the elimination of pre-malignant cells *in vivo*. Pharmacologic agents that selectively enhance apoptosis have been shown to lower the risk of a variety of cancers in animals and in humans. (107-108) In addition, in both animal models of cancer as well as in humans, the efficacy of cancer preventive agents has been shown to correlate with the degree of apoptosis induced. (22, 107, 109, 110)

Conversely, mutations in the genes involved in the apoptosis pathway have been shown to be associated with enhanced cancer risk. (110)

In light of the known association between activation of TGF-β and apoptosis molecular pathways and cancer prevention, the observation that progestins markedly activate these pathways in the endometrium opens the door toward development of a progestin-based strategy for the effective chemoprevention of endometrial cancer. Moreover, given that progestins have similar chemopreventive biologic effects in both the ovary and the endometrium, it is interesting to speculate that a potent progestin-based strategy may effectively prevent both ovarian and uterine cancer, thereby decreasing the incidence and mortality from the two most common gynecologic malignancies.

References

1) Amant F, Moerman P, Neven P, Timmerman D, Limbergan E, Vergote I. Endometrial Cancer. Lancet 2005;366(9484):491-505.
2) American Cancer Society (ACS). 2006. *Cancer Facts and Figures 2006.* Atlanta: American Cancer
3) Henderson BE, Casagrance JT, Pike MC, et al. The epidemiology of endometrial cancer in young women. British Journal of Cancer 1983; 47:749-756.
4) McPherson CP, Sellers TA, Potter JD, Bostick RM, Folsom AR. Reproductive factors and risk of endometrial cancer. The Iowa women's health study. *American Journal of Epidemiology* 1996;143:1195-1202
5) The Writing Group for the PEPI trial. Effects of hormone replacement therapy on endometrial histology in postmenopausal women: the Postmenopausal Estrogen/ Progestin Interventions (PEPI) Trial. JAMA 1996;275:370-5.
6) Xu W, Xiang Y-B, ZhengWet al.Weight history and risk of endometrial cancer among Chinese women. Int J Epidemiol 2006;35:159–66.
7) Trentham-Dietz A, Nichols H, Hampton J, Newcomb P. Weight change and risk of endometrial cancer. Int J Epidemiol 2006;35:151–58.

8) Weiderpass E, Persson I, Adami HO, Magnusson C, Lindgren A, Baron JA. Body size in different periods of life, diabetes mellitus, hypertension, and risk of postmenopausal endometrial cancer (Sweden). Cancer Causes Control 2000;11:185–92.
9) Shulman LP, Yankov V, Uhl K. Safety and efficacy of a continuous once-a-week 17β-estradiol/levonorgestrel transdermal system and its effects on vasomotor symptoms and endometrial safety in postmenopausal women: the results of two multicenter, double-blind, randomized, controlled trials. *Menopause* 2002; 9:195-207
10) Penelope M. Webb. Commentary: Weight gain, weight loss, and endometrial cancer. International Journal of Epidemiology 2006;35:166–168
11) Vessey M and Painter R. Oral contraceptive use and cancer. Findings in a large cohort study, 1968-2004. *British Journal of Cancer* 2006; 95:385-389.
12) Hulka BS, Chambless LE, Kaufman DG, Fowler WC, Greenberg BG. Protection against endometrial carcinoma by combination-product oral contraceptives. *Journal of the American Medical Association* 1982; 247:475-477.
13) The Cancer and Steroid Hormone (CASH) Study of the Centers for Disease Control and the National Institute of Child Health and Human Development. Combination oral contraceptive use and the risk of endometrial cancer. *The Journal of the American Medical Association* 1987; 257:796-800.
14) The WHO collaborative study of neoplasia and steroid contraceptives. Endometrial cancer and combined oral contraceptives. *International Journal of Epidemiology* 1988;17:263-269
15) Jick SS, Walker AM, Jick H. Oral contraceptives and endometrial cancer. *Obstetrics & Gynecology* 1993; 82:931-935.
16) Maxwell GL, Schildkraut JM, Calingaert B, Risinger JI, Dainty L, Marchbanks PA, Berchuck A, Barrett JC, Rodriguez GC. Progestin and estrogen potency of combination oral contraceptives and endometrial cancer risk. *Gynecologic Oncology* (Epub Gynecol Oncol doi:10.1016/j.ygyno.2006.03.046).
17) Rosenblatt KA, Thomas DB, WHO Collaborative Study of Neoplasia and Steroid Contraceptives. Hormonal content of combined oral contraceptives in relation to the reduced risk of endometrial carcinoma. *International Journal of Cancer* 1991; 49:870-874.
18) Benshushan A, Paltiel O, Rojansky N, Brzezinski A, Laufer N. IUD use and the risk of endometrial cancer. *European Journal of Obstetrics & Gynecology and Reproductive Biology* 2002; 105:166-169.
19) Tao MH, Xu WH, Zheng W, Zhang ZF, Gao YT, Ruan ZX, Cheng JR, Gao J, Xiang YB, Shu XO. Oral contraceptive and IUD use and endometrial cancer: A population-based case-control study in Shanghai, China. *International Journal of Cancer* (Epub 5 July 2006).
20) Orbo A, Rise CE, Mutter G. Regression of latent endometrial precancers by progestin infiltrated intrauterine device. Cancer Research 2006; 66: 5613-5617.
21) Randall TC, Kurman RJ. Progestin treatment of atypical hyperplasia and well-differentiated carcinoma of the endometrium in women under age 40. Obstet Gynecol 1997 ;90(3):434–40.

22) Vereide AB, Kaino T, Sager G, Ørbo A. Bcl-2, BAX, and apoptosis in endometrial hyperplasia after high dose gestagen therapy: A comparison of responses in patients treated with intrauterine levonorgestrel and systemic medroxyprogesterone. *Gynecologic Oncology* 2005; 97:740-750.
23) Rodriguez GC, Nagarsheth NP, Lee KL, et al. Progestin-induced apoptosis in the macaque ovarian epithelium: Differential regulation of transforming growth factor-beta. *J* Natl Cancer Inst. 2002;94:50-60.
24) Booth M, Beral V, Smith P. Risk factors for ovarian cancer: A case-control study. *British Journal of Cancer* 1989; 60:592-598.
25) Greer JB, Modugno F, Allen GO, Ness RB. Short-term oral contraceptive use and the risk of epithelial ovarian cancer. *American Journal of Epidemiology* 2005; 162:66-72.
26) Parazzini F, La Vecchia C, Negri E, Bocciolone L, Fedele L, Franceschi S. Oral contraceptive use and the risk of ovarian cancer: An Italian case-control study. *European Journal of Cancer* 1991b; 27:594-598.
27) Rosenberg L, Shapiro S, Slone D, Kaufman DW, Helmrich SP, Miettinen OS, Stolley PD, Rosenshein NB, Schottenfeld D, Engle RL. Epithelial ovarian cancer and combination oral contraceptives. *The Journal of the American Medical Association* 1982; 247:3210-3212.
28) Schildkraut JM, Calingaert B, Marchbanks PA, Moorman PG, Rodriguez GC. Impact of progestin and estrogen potency in oral contraceptives on ovarian cancer risk. *Journal of the National Cancer Institute* 2002; 94:32-38.
29) The WHO collaborative study of Neoplasia and Steroid Contraceptives. Epithelial ovarian cancer and combined oral contraceptives. *International Journal of Epidemiology* 1989; 18:538-545.
30) Whittemore AS, Harris R, Itnyre J. Characteristics relating to ovarian cancer risk: Collaborative analysis of 12 US case-control studies. IV. The pathogenesis of epithelial ovarian cancer. Collaborative Ovarian Cancer Group. *Am J Epidemiol.* 1992;136:1212-1220.
31) Weiss NS, Cook LS, Farrow DC, Rosenblatt KA. Ovarian cancer. In: Schottenfeld D, Fraumeni JF Jr, eds. *Cancer Epidemiology and Prevention*. New York (NY): Oxford University Press; 1996;1040-57.
32) Rosenberg L, Palmer JR, Zauber AG, et al. A case-control study of oral contraceptive use and invasive ovarian cancer. *Am J Epidemiol.* 1994:139:654-61.
33) Anonymous. The reduction in risk of ovarian cancer associated with oral-contraceptive use. The Cancer and Steroid Hormone Study of the Centers for Disease Control and the National Institute of Child Health and Human Development. *N Engl J Med.* 1987;316:650-655.
34) Risch HA, Weiss NS, Lyon JL, Daling JR, Liff JM. Events of reproductive life and the incidence of epithelial ovarian cancer. *Am J Epidemiol.* 1983;117;128-39.
35) Narod SA, Risch H, Moslehi R, et al. Oral contraceptives and the risk of hereditary ovarian cancer. Hereditary Ovarian Cancer Clinical Study Group. *N Engl J Med.* 1998;339:424-428.
36) R.J. Kurman (Ed.): Blaustein's Pathology of the Female genital Tract, 5th ed.,Springer-Verlag, New York, 2002.

37) Cline JM, Söderqvist G, Register TC, Williams JK, Adams MR, Von Schoultz B. Assessment of hormonally active agents in the reproductive tract of female nonhuman primates. Toxicol Pathol 2001;29(1):84-90.
38) Brenner RM, Slayden OD. Cyclic changes in the primate oviduct and endometrium. In: Knobil E, Neill JD, editors. The physiology of reproduction. New York (NY): Raven Press; 1994. p. 541–69.
39) Hotchkiss J, Knobil E. The menstrual cycle and its neuroendocrine control. In: Knobil E, Neill JD, editors. The physiology of reproduction. New York (NY): Raven Press; 1994. p. 711–36.
40) Kaiserman-Abramof IR, Padykula HA. Ultrastructural epithelial zonation of the primate endometrium (rhesus monkey). Am J Anat 1989;184: 13–30.
41) Cline JM, Söderqvist G, von Schoultz E, Skoog L, von Schoultz B. Effects of conjugated estrogens, medroxyprogesterone acetate, and tamoxifen on the mammary glands of macaques. Breast Cancer Res Treat 1998;48:221-229.
42) Conner P, Register TC, Skoog L, Tani E, von Schoultz Cline JM. Expression of p53 and markers for apoptosis in breast tissue during long-term hormone therapy in cynomolgus monkeys. American Journal of Obstetrics & Gynecology. 2005; 193(1):58-63,
43) Amezcua CA. Lu JJ. Felix JC. Stanczyk FZ. Zheng W. Apoptosis may be an early event of progestin therapy for endometrial hyperplasia. Gynecologic Oncology. 2000; 79(2):169-76.
44) Akcali KC. Khan SA. Moulton BC. Effect of decidualization on the expression of bax and bcl-2 in the rat uterine endometrium. Endocrinology. 1996; 137(7):3123-31.
45) Hanifi-Moghaddam P. Gielen SC. Kloosterboer HJ. De Gooyer ME. Sijbers AM. van Gool AJ. Smid M. Moorhouse M. van Wijk FH. Burger CW. Blok LJ. Molecular portrait of the progestagenic and estrogenic actions of tibolone: behavior of cellular networks in response to tibolone. Journal of Clinical Endocrinology & Metabolism. 2005; 90(2):973-83.
46) Wang S. Pudney J. Song J. Mor G. Schwartz PE. Zheng W. Mechanisms involved in the evolution of progestin resistance in human endometrial hyperplasia--precursor of endometrial cancer. Gynecologic Oncology. 2003; 88(2):108-17.
47) Maruo T, Laoag-Fernandez JB, Pakarinen P, Murakoshi H, Spitz IM, Johansson E. Effects of the levonorgestrel-releasing intrauterine system on proliferation and apoptosis in the endometrium. Hum Reprod 2001;16:2103–8
48) Vaskivuo TE, Stenback F, Karhumaa P, Risteli J, Dunkel L, Tapanainen JS. Apoptosis and apoptosis-related proteins in human endometrium. Mol Cell Endocrinol 2000;165:75– 83
49) Reis FM. Ribeiro MF. Maia AL. Spritzer PM. Regulation of human endometrial transforming growth factor beta1 and beta3 isoforms through menstrual cycle and medroxyprogesterone acetate treatment. Histology & Histopathology. 2002; 17(3):739-45.
50) Rosario GX. Sachdeva G. Manjramkar DD. Modi DN. Meherji PK. Puri CP. Endometrial expression of immunomodulatory cytokines and their regulators

during early pregnancy in bonnet monkeys (Macaca radiata). Human Reproduction. 2005; 20(11):3039-46.
51) Okulicz WC. Ace CI. Longcope C. Tast J. Analysis of differential gene regulation in adequate versus inadequate secretory-phase endometrial complementary deoxyribonucleic acid populations from the rhesus monkey. Endocrinology. 1996; 137(11):4844-50.
52) Ace CI. Okulicz WC. Differential gene regulation by estrogen and progesterone in the primate endometrium. Molecular & Cellular Endocrinology. 115(1):95-103, 1995
53) Yang NN, Bryant HU, Hardikar S, Sato M, Galvin RJ, Glasebrook AL, et al. Estrogen and raloxifene stimulate transforming growth factor-beta 3 gene expression in rat bone: a potential mechanism for estrogen of raloxifene-mediated bone maintenance. Endocrinology 1996;137:2075–84.
54) Arrick BA, Kore M, Derynck R. Differential regulation of expression of three transforming growth factor beta species in human breast cancer cell lines by estradiol. Cancer Res 1990;50:299–303.
55) Butta A, MacLennan K, Flanders KC, Sacks NP, Smith I, McKinna A, et al. Induction of transforming growth factor _1 in human breast cancer *in vivo* following tamoxifen treatment. Cancer Res 1992;52:4261–4.
56) Farquharson C, Law AS, Seawright E, Burt DW, Whitehead CC. The expression of transforming growth factor-beta by cultured chick growth plate chondrocytes: differential regulation by 1,25-dihydroxyvitamin D3. J Endocrinol 1996;149:277–85.
57) Frank S, Madlener M, Werner S. Transforming growth factors beta1, beta2, and beta3 and their receptors are differentially regulated during normal and impaired wound healing. J Biol Chem 1996;271:10188–93.
58) Degitz SJ, Morris D, Foley GL, Francis BM. Role of TGF-beta in RA-induced cleft palate in CD-1 mice. Teratology 1998;58:197–204.
59) Roberts AB, Sporn MB. Mechanistic interrelationships between two superfamilies: the steroid/retinoid receptors and transforming growth factor- β. Cancer Surv 1992;14:205–20.
60) Wakefield L, Kim SJ, Glick A, Winokur T, Colletta A, Sporn M. Regulation of transforming growth factor-_ subtypes by members of the streroid hormone superfamily. J Cell Sci Suppl 1990;13:139–48.
61) Desai KV, Kondaiah P. Androgen ablation results in differential regulation of transforming growth factor-beta isoforms in rat male accessory sex organs and epididymis. J Mol Endocrinol 2000;24:253–60.
62) Geiser AG, Busam KJ, Kim SJ, Lafyatis R, O'Reilly MA, Webbink R, et al. Regulation of the transforming growth factor-beta 1 and -beta 3 promoters by transcription factor Sp1. Gene 1993;129:223–8.
63) Kim SJ, Park K, Koeller D, Kim KY, Wakefield LM, Sporn MB, et al. Post-transcriptional regulation of the human transforming growth factorbetagene. J Biol Chem 1992;267:13702–7.
64) Kim SJ, Glick A, Sporn MB, Roberts AB. Characterization of the promoter region of the human transforming growth factor-beta 1 gene. J Biol Chem 1989;264:402–8.

65) Noma T, Glick AB, Geiser AG, O'Reilly MA, Miller J, Roberts AB, et al. Molecular cloning and structure of the human transforming growth factorbeta 2 gene promoter. Growth Factors 1991;4:247–55.
66) Haufel T, Dormann S, Hanusch J, Schwieger A, Bauer G. Three distinct roles for TGF-_ during intercellular induction of apoptosis: a review.Anticancer Res 1999;19:105–12.
67) Bursch W, Oberhammer F, Jirtle RL, Askari M, Sedivy R, Grasl-Kraupp B, et al. Transforming growth factor- β1 as a signal for induction of cell death by apoptosis. Br J Cancer 1993;67:531–6.
68) Chiarugi V, Magnelli L, Cinelli M. Complex interplay among apoptosis factors: RB, p53, E2F, TGF-β, cell cycle inhibitors and the bcl2 gene family. Pharmacol Res 1997;35:257–61.
69) Berchuck A, Rodriguez G, Olt GJ, Bent MP, Whitaker R, Arrick B, Clarke-Pearson DL, Bast RC Jr: Regulation of growth of normal ovarian epithelial cells and ovarian cancer cell lines by transforming growth factor-beta. Am J Obstet Gynecol 1992; 166:676-84.
70) Hurteau J, Rodriguez GC, Whitaker RS, Shah S, Mills G, Bast RC Jr, Berchuck A: Transforming growth factor-β inhibits proliferation of human ovarian cancer cells obtained from ascites. Cancer 1994; 74:93-9.
71) Havrilesky LJ, Hurteau JA, Whitaker RS, Elbendary A, Wu S, Rodriguez GC, Bast RC Jr., Berchuck A: Regulation of apoptosis in normal and malignant ovarian epithelial cells by transforming growth factor beta. Cancer Research 1995; 55:944-948.
72) Rotello RJ, Lieberman RC, Purchio AF, Gershenson LE. Coordinated regulation of apoptosis and cell proliferation by transforming growth factor 1 in cultured uterine epithelial cells. Proc Natl Acad Sci U S A 1991;88:3412–5.
73) El Etreby MF, Liang Y, Wrenn RW, Schoenlein PV. Additive effect of mifepristone and tamoxifen on apoptotic pathways in MCF-7 human breast cancer cells. Breast Cancer Res Treat 1998;51:149–68.
74) James SY, Mackay AG, Colston KW. Effects of 1,25 dihydroxyvitamin D3 and its analogues on induction of apoptosis in breast cancer cells. J Steroid Biochem Mol Biol 1996;58:395–401.
75) Formby B, Wiley TS. Progesterone inhibits growth and induces apoptosis in breast cancer cells: inverse effects on Bcl-2 and p53. Ann Clin Lab Sci 1998;28:360–9.
76) Strange R, Li F, Saurer S, Burkhardt A, Friis RR. Apoptotic cell death and tissue remodeling during mouse mammary gland involution. Development 1992;115:49–58.
77) Kyprianou N, Isaacs JT. Expression of transforming growth factor-beta in the rat ventral prostate during castration-induced programmed cell death. Mol Endocrinol 1989;3:1515–22.
78) Roberson KM, Penland SN, Padilla GM, Selvan RS, Kim CS, Fine RL, et al. Fenretinide: induction of apoptosis and endogenous transforming growth factor-β in PC-3 prostate cancer cells. Cell Growth Differ 1997; 8:101–11.
79) Colletta AA, Wakefield LM, Howell FV, Danielpour D, Baum M, Sporn MB. The growth inhibition of human breast cancer cells by a novel synthetic progestin

involves the induction of transforming growth factor beta. J Clin Invest 1991;87:277–83.
80) Rendi MH, Suh N, Lamph WW, Krajewski S, Reed JC, Heyman RA, Berchuck A, Liby K, Risingsong R, Royce DB, Williams CR, Sporn MB. The selective estrogen receptor modulator arzoxifene and the rexinoid LG100268 cooperate to promote transforming growth factor beta-dependent apoptosis in breast cancer. Cancer Research 2004; 64:3566-3571
81) Roznovanu SL, Amalinci C, Radulescu D. Molecular mechanisms in hormone-resistant prostate cancer. Revista Medico-Chirurgicala a Societatii de Medici Si Naturalisti Din Iasi 2005; 109: 577-83
82) Tu Y, Jerry DJ, Pazik B, Smith Schneider S. Sensitivity to DNA damage is a common component of hormone-based strategies for protection of the mammary gland. Molecular Cancer Research 2005; 3: 435-42.
83) Zarzynska J, Gajewska M, Tomasz M. Effects of hormones and growth factors on TGF-beta 1 expression in bovine mammary epithelial cells. Journal of Dairy Research 2005; 72: 39-48.
84) Pollard JW. Tumor-stromal interactions. Transforming Growth Factor –Beta isoforms and hepatocyte growth factor/scatter factor in mammary gland ductal morphogenesis. Breast Cancer Research 2001; 3: 230-7.
85) Mishina Y, Whitworth DJ, Racine C, Behringer RR. High specificity of Müllerian-inhibiting substance signaling *in vivo.* Endocrinology 1999; 140:2084–8.
86) Voutilainen R, Miller WL. Potential relevance of mullerian-inhibiting substance to ovarian physiology. Semin Reprod Endocrinol 1989;7: 88–93.
87) Cate RL, Mattaliano RJ, Hession C, Tizard R, Farber NM, Cheung A, et al. Isolation of the bovine and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. Cell 1986; 45:685–98.
88) Byskov AG. Differentiation of mammalian embryonic gonad. Physiol Rev 1986;66:71–117.
89) Masiakos PT, MacLaughlin DT, Maheswaran S, Teixeira J, Fuller AF Jr, Shah PC, Kehas DJ, Kenneally MK, Dombkowski DM, Ha TU, Preffer FI, Donahoe PK. Human ovarian cancer, cell lines, and primary ascites cells express the human Mullerian inhibiting substance (MIS) type II receptor, bind, and are responsive to MIS. Clin Cancer Res. 1999 (11):3488-99.
90) Ha TU, Segev DL, Barbie D, Masiakos PT, Tran TT, Dombkowski D, Glander M, Clarke TR, Lorenzo HK, Donahoe PK, Maheswaran S. Mullerian inhibiting substance inhibits ovarian cell growth through an Rb-independent mechanism. J Biol Chem. 2000 Nov 24;275(47):37101-9.
91) Stephen AE, Pearsall LA, Christian BP, Donahoe PK, Vacanti JP, MacLaughlin DT. Highly purified mullerian inhibiting substance inhibits human ovarian cancer in vivo. Clin Cancer Res. 2002 Aug;8(8):2640-6.
92) Stephen AE, Masiakos PT, Segev DL, Vacanti JP, Donahoe PK, MacLaughlin DT. Tissue-engineered cells producing complex recombinant proteins inhibit ovarian cancer in vivo. Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):3214-9. Epub 2001 Feb 27.

93) Renaud EJ. MacLaughlin DT. Oliva E. Rueda BR. Donahoe PK. Endometrial cancer is a receptor-mediated target for Mullerian Inhibiting Substance. Proc Natl Acad Sci U S A. 2005; 3;102:111-116
94) Sporn MB, Roberts AB, Wakefiled LM, Glick AB, Danielpour D. Transforming Growth Factor Beta and suppression of carcinogenesis. Princess Takamatsu Symposia 1989; 20: 259-266
95) Akhurst RJ, Balmain A. Genetic events and the role of TGF beta in epithelial tumour progression. J Pathol 1999;187:82–90.
96) Reiss M. Transforming growth factor- β and cancer: a love–hate relationship? Oncol Res 1997;9:447–57.
97) Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor in human disease. N Engl J Med 2000;342:1350–8.
98) Wakefield LM, Yang YA, Dukhania O. Transforming Growth Factor Beta and breast cancer: lessons learned from genetically altered mouse models. Breast Cancer Research 2000; 2: 100-6
99) Narayan S, Thangasamy T, Balusu R. Transforming Growth Factor Beta signaling in cancer. Frontiers in Bioscience 2005; 10; 1135-45
100) Pierce DF Jr, Gorska AE, Chytil A, Meise KS, Page DL, Coffey RJ Jr, et al. Mammary tumor suppression by transforming growth factor beta 1 transgene expression. Proc Natl Acad Sci U S A 1995;92:4254–8.
101) Tang B, Bottiner E, Bagnall K, Anver M, Wakefield L. Enhanced liver and lung tumorigenesis in TGF-_1 heterozygous knock-out mice following treatment with diethylnitrosamine and phenobarbital [abstract]. Proc Am Assoc Cancer Res 1997;38:584.
102) Lin HK, Bergmann S, Pandolfi PP. Deregulated TGF-Beat signaling in leukemogenesis. Oncogene 2005:24; 5693-5700.
103) Izzi L, Attisano L. Ubiquitin-dependent regulation of TGF-beta signalling in cancer. Neoplasia 2006; 8: 677-688.
104) Elliot RL, Blobe GC. Role of Transforming Growth Factor Beta in human cancer. Journal of Clinical Oncology 2005; 23; 2078-2093.
105) Kaklamani VG, Pasche B. Role of TGF-Beta in cancer and the potential for therapy and prevention. Expert Review of Anticancer Therapy 2004; 4:649-61.
106) Park SH. Fine tuning and cross-talking of TGF-Beta signal by inhibitory smads. Journal of Biochemistry and Molecular Biology 2005; 38; 9-16
107) Sun SY, Hail N, Lotan R. Apoptosis as a novel target for cancer prevention. J Natl Cancer Inst: 2004; 96:662-72.
108) Ouyang, Nengtai. Williams, Jennie L. Rigas, Basil. NO-donating aspirin isomers downregulate peroxisome proliferator-activated receptor (PPAR)delta expression in APC(min/+) mice proportionally to their tumor inhibitory effect: Implications for the role of PPARdelta in carcinogenesis. Carcinogenesis. 2006; 27(2):232-9.
109) Sinicrope FA. Half E. Morris JS. Lynch PM. Morrow JD. Levin B. Hawk ET. Cohen DS. Ayers GD. Stephens LC. Cell proliferation and apoptotic indices predict adenoma regression in a placebo-controlled trial of celecoxib in

familial adenomatous polyposis patients. Cancer Epidemiology, Biomarkers & Prevention. 2004; 13(6):920-7.

110) Reed JC. Mechanisms of apoptosis avoidance in cancer. Curr Opin Oncol 1999; 11:68–75.

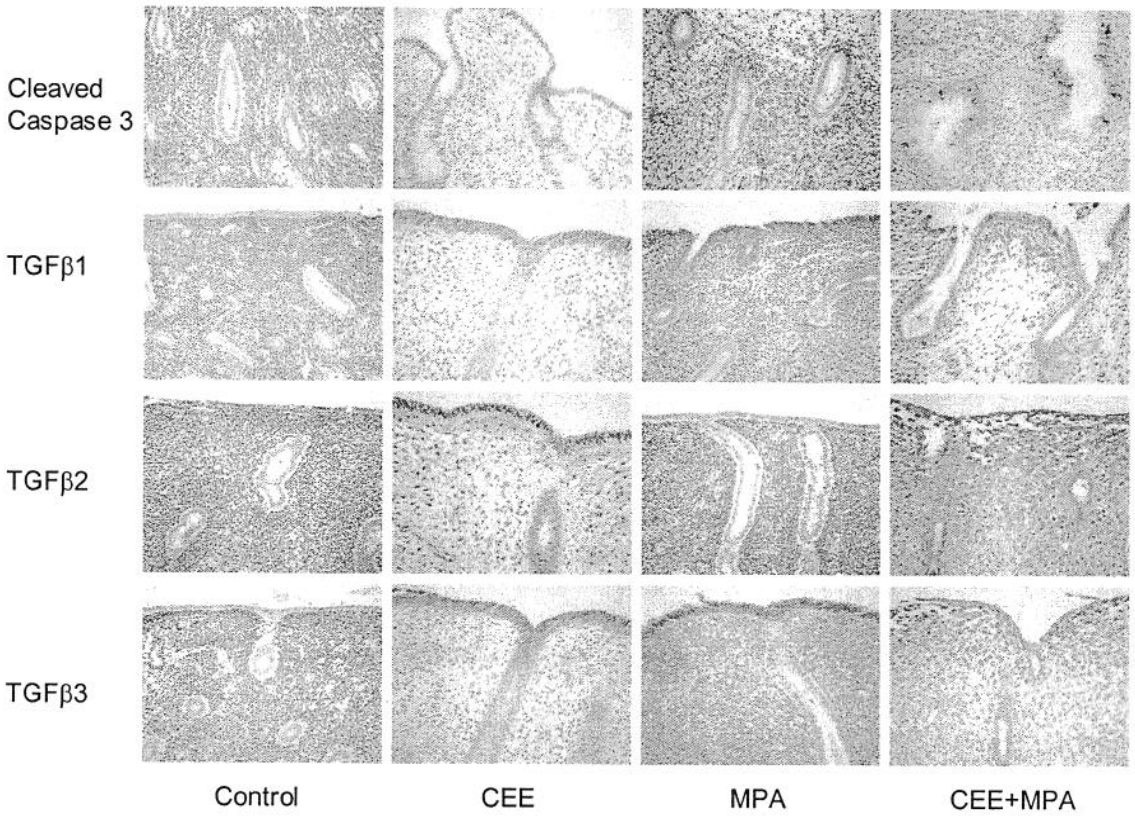

Figure 1. Representative sections from each of the treatment groups, immunostained for activated caspase-3 and the TGF-β isoforms.

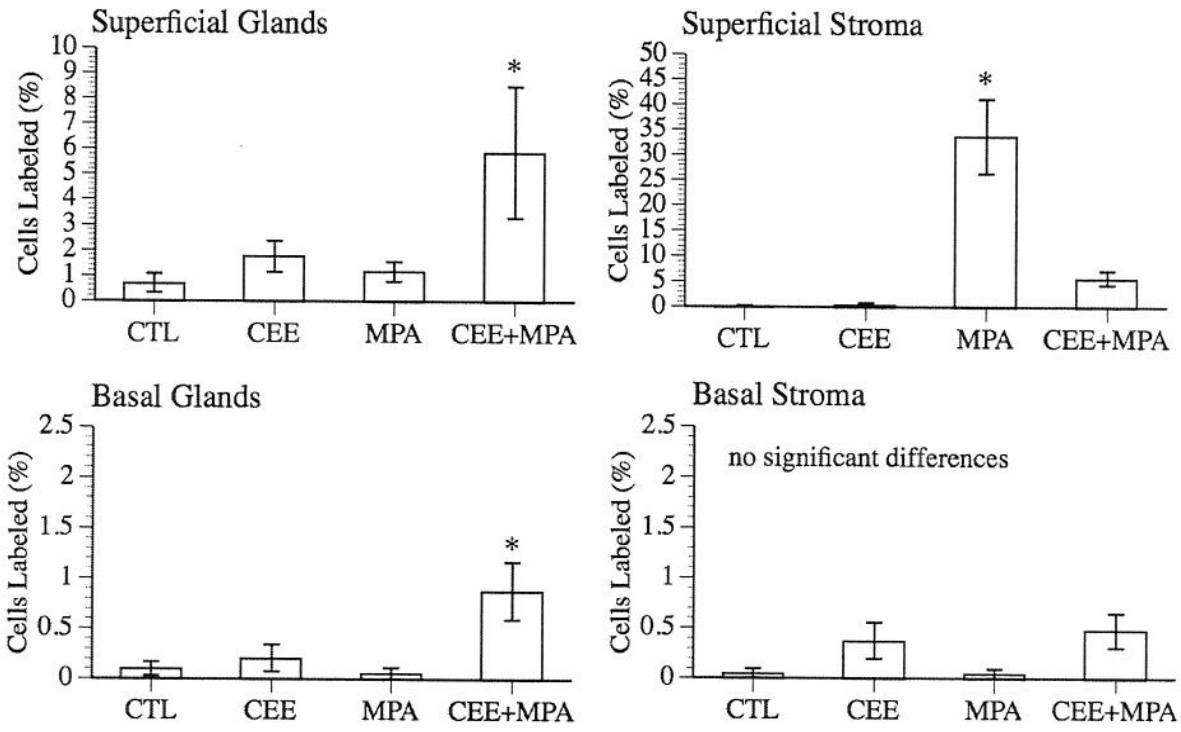

Figure 2: Cleaved caspase-3 immunostaining expressed as a percentage of cells counted in glandular and stromal compartments of the superficial and basal endometrium. Asterisks indicate groups whose means differ from controls at $p<0.05$

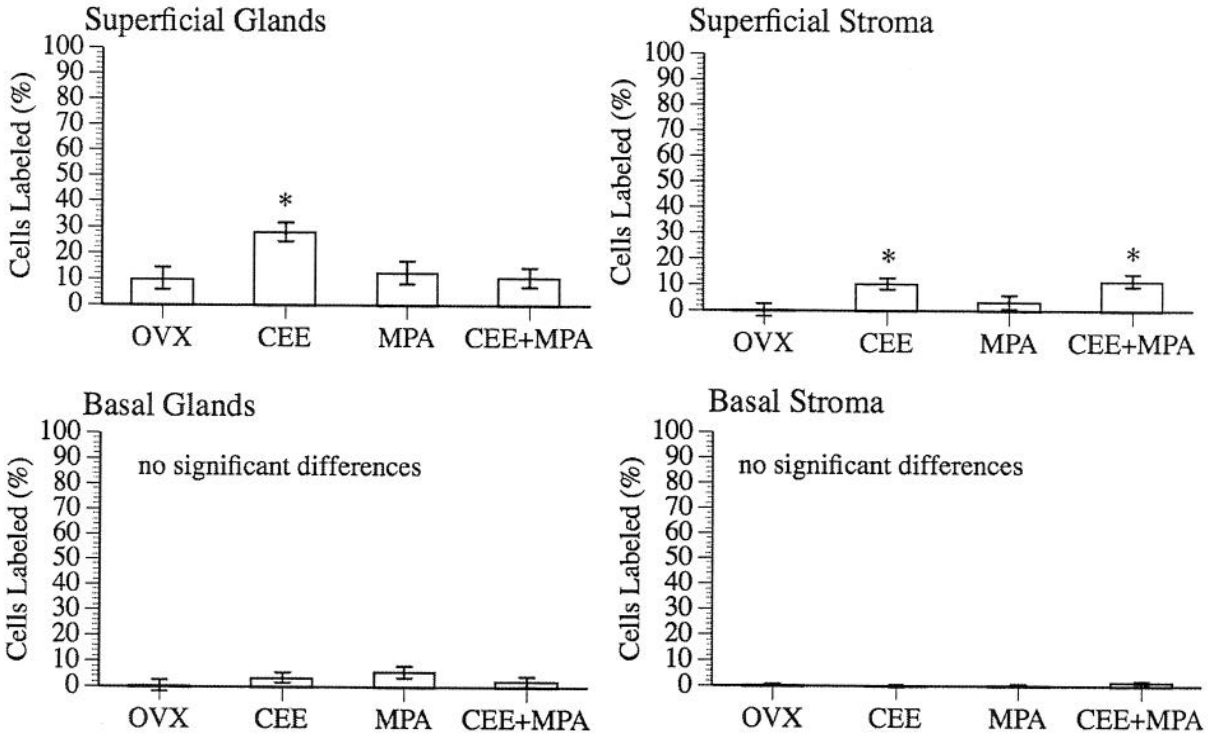

Figure 3. Ki67 immunostaining expressed as percent cells labeled. Asterisks indicate groups whose means differ from controls at $p<0.05$

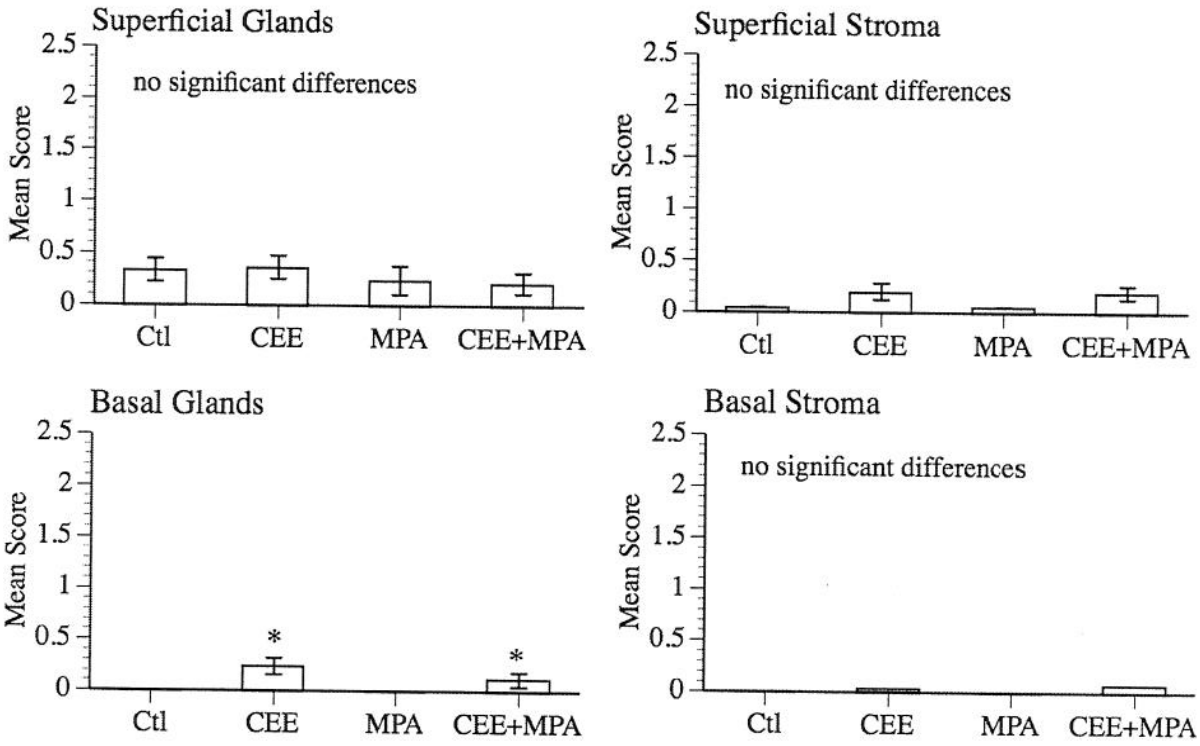

Figure 4: TGF-β1 immunostaining expressed as mean staining score in surface epithelium and glandular and stromal compartments of the superficial and basal endometrium. Asterisks indicate groups whose means differ from controls at $p<0.05$. In Basal Glands, immunostaining in the CEE and CEE+MPA groups was greater than controls ($p= 0.009$ and 0.039, respectively).

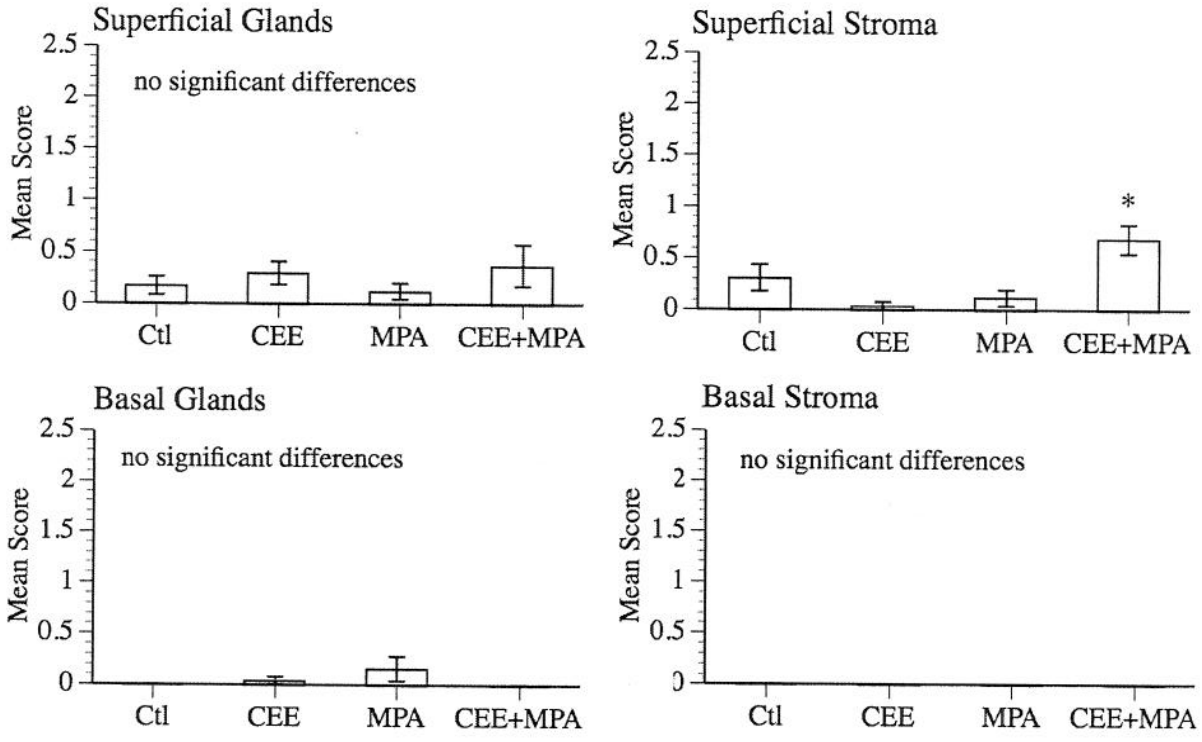

Figure 5: TGF-β2 immunostaining expressed as mean staining score in surface epithelium and glandular and stromal compartments of the superficial and basal endometrium. Asterisks indicate groups whose means differ from controls at $p<0.05$

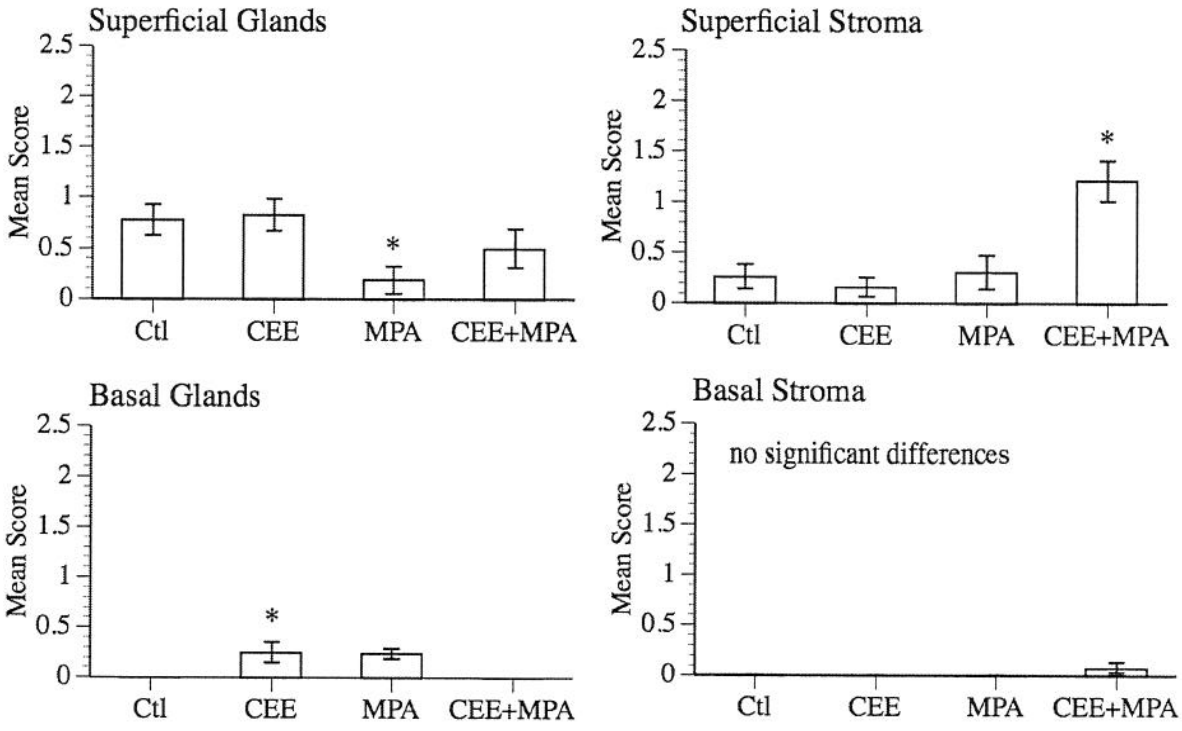

Figure 6: TGF-β3 immunostaining expressed as mean staining score in surface epithelium and glandular and stromal compartments of the superficial and basal endometrium. Asterisks indicate groups whose means differ from controls at $p<0.05$. In Superficial Glands, immunostaining in the MPA group was significantly lower than both control and CEE.